phospholipid interactions may play a key role in viral maturation and budding from the cytoplasmic membrane of the infected host cell.

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Photoaffinity Labeling of a Synaptic Vesicle Specific Nucleotide Transport System from *Torpedo marmorata*†

Deborah Anne Lee and Veit Witzemann*

ABSTRACT: We have employed azido derivatives of ATP and AMP to identify the ATP translocase of synaptic vesicles. Azido-AMP inhibits transport of both ATP and AMP in vitro. The affinity of the translocase for the azido derivatives is similar to that of the native ligands. Upon UV irradiation of vesicles incubated with radiolabeled azido-AMP or -ATP, a molecular weight (M_r) 34 000 polypeptide is selectively modified. On two-dimensional gel electrophoresis, the single radiolabeled polypeptide has a p*I* of ~7.7. Analysis of the fractions obtained when vesicles were purified on linear sucrose density gradients reveals that the M_r 34 000 polypeptide is highly enriched in the vesicle-containing fractions. The findings support the notion that this polypeptide is identical with a previously described vesicle-specific component of the same molecular size [Stadler, H., & Tashiro, T. (1979) *Eur. J. Biochem. 101*, 171–178], and we conclude on the basis of uptake inhibition and photoaffinity labeling results that this protein is directly involved in ATP translocation of synaptic vesicles.

Synaptic vesicles and vesicles from endocrine glands which act as storage sites and vehicles for the release of neurotransmitters and hormones often contain high concentrations of nucleotides (Winkler, 1977; de Potter et al., 1970). For example, cholinergic synaptic vesicles from the electromotor tissue of *Torpedo marmorata* have been shown to contain molar quantities of adenosine 5′-triphosphate (ATP) (Dowdall et al., 1974), which is released upon stimulation along with acetylcholine. Subsequent reuptake of the ATP into the newly formed vesicle population parallels that of acetylcholine (Zimmermann, 1979). Although the function of this large store of vesicular ATP is not known, modifications of transmitter response by adenosine nucleotides have been documented (Israel & Meunier, 1978; McAfee & Greengard, 1972).

The transport of ATP into synaptic vesicles has been shown

† From the Max-Planck-Institut für biophysikalische Chemie, Abteilung Neurochemie, D-3400 Göttingen, West Germany. *Received May 18, 1983.* Financial support for this research was obtained from the Max-Planck-Gesellschaft and the Deutsche Forschungsgemeinschaft. D.A.L. received an Auslandsstipendium from the Max-Planck-Gesellschaft.

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to proceed in vitro by a carrier-mediated process with general specificity, transporting ATP, ADP, and AMP with similar efficiencies (Luqmani, 1981). This implies the presence of a specific nucleotide transport protein associated with the synaptic vesicles. Highly purified vesicle preparations have been shown to be enriched in a number of distinct proteins which are thought to be specific components of the vesicles (Tashiro & Stadler, 1978; Stadler & Tashiro, 1979; Wagner et al., 1978). A prominent membrane component is vesicle protein 11 (V11) (Tashiro & Stadler, 1978), a basic protein of molecular weight (M_r) 34 000. Antibodies raised to this protein stimulate the uptake of ATP by isolated synaptic vesicles in vitro (Luqmani, 1981), and there is evidence that palmitoyl coenzyme A (palmitoyl-CoA) which inhibits ATP uptake in vitro (Luqmani, 1980) binds to this protein (Fenwick & Stadler, 1981). In order to further characterize the carrier-mediated transport system, we sought to identify the polypeptide(s) involved in nucleotide transport in synaptic vesicles.

Photoactivated nucleotide analogues have been successfully used to identify and characterize proteins such as the mitochondrial ADP/ATP translocase (Schafer et al., 1976), the F_1-ATPase (Wagenvoord et al., 1977), and mouse ribonucleotide reductase (Caras & Martin, 1982).

We demonstrate that nucleotide transport by isolated synaptic vesicles can be specifically inhibited by using azido derivatives of ATP and AMP. The same nucleotide analogues are shown to selectively modify a vesicle polypeptide of M_r 34 000 upon irradiation. These experiments allow the assignment of a functional property of synaptic vesicles to a biochemically identified protein component of the vesicular membrane.

Materials and Methods

Materials

Chemicals. The photoaffinity probes azido[^{3}H]AMP (8-azido[2-^{3}H]adenosine 5′-monophosphate) and azido[^{32}P]ATP (8-azidoadenosine 5′-[α-^{32}P]triphosphate) and radiolabeled compounds were obtained from New England Nuclear (FRG). Unlabeled azido-AMP (8-azidoadenosine 5′-monophosphate) was from Sigma (Munich).

Animals. Experiments were performed by using female specimens of *Torpedo marmorata*, supplied by the Station Biologique d'Arcachon, France, and kept in tanks of circulating artificial seawater maintained at 15–18 °C. Electric organs were removed from anesthetized fish and stored frozen in liquid nitrogen.

Methods

Preparative Isolation of Synaptic Vesicles on a Linear Sucrose Gradient. Liquid nitrogen frozen electromotor tissue, after crushing into a coarse powder, was thawed to −2 °C and taken up (1:2 frozen weight:volume) into glycine (0.85 M) buffered with 5 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes), pH 6.9 (buffer A), and the resulting slurry was squeezed through four to six layers of cheesecloth. The filtrate was centrifuged for 20 min at 12000*g* to remove large subcellular fragments including mitochondria (crude mitochondrial pellet); the supernatant thus obtained was respun at 60000*g* for 15 h (Beckman SW27 rotor). After being loaded onto a linear isoosmotic gradient of buffered sucrose/glycine [from top to bottom, 0.64 M sucrose/0.85 M glycine (1:2) to 0.64 M sucrose], the gradient, 15 mL/tube, was layered onto a 2-mL cushion of 1.6 M sucrose to facilitate subsequent fractionation with a Beckman fraction collector, and vesicles were located by their ATP content. This gradient is fully characterized elsewhere (Giompres et al., 1981) and is slightly modified from the procedure of Breer et al. (1977). In the peak vesicle fraction, the presence of vesicles was established by electron microscopy and immunochemistry. No fumarase activity was observed, and less than 0.3% of the total lactate dehydrogenase activity was found in the vesicle fraction. Cytochrome *c* oxidase activity was less than 4% of the activity found in the crude mitochondrial-containing pellet. Collected vesicles were diluted with buffer A (100–200 μg of protein/mL) and used within 4 h of preparation for the transport studies. Vesicles used for the labeling experiments were frozen in buffer A and thawed prior to use.

Uptake Studies. Vesicle preparations (100–200 μg of protein/mL) in 0.85 M glycine, 5 mM Hepes, pH 6.9, and 1 mM AMP were incubated with various concentrations of azido-AMP for 30 min at 4 °C. The suspensions were irradiated with UV light (254 nm) for 2 min on ice. Irradiation was performed with a UV lamp (Universal UV Lampe, Typ TL-900, Camag, West Berlin) at a distance of 7 cm. Radiolabeled [^{3}H]AMP was then added (20 μM), and uptake experiments were performed as described (Luqmani, 1981).

Irreversible Inhibition of [^{3}H]AMP Uptake. Five hundred microliters of vesicles (~200 μg of protein) was photolyzed (2 min at 254 nm on ice) in the presence of N_3AMP. Immediately after photolysis, the vesicles were separated by gel filtration through a short Sephadex G-50 column (5 × 0.5 cm) from excess photolabel. The vesicles eluted with the void volume in ca. 0.8 mL retained about 5% of the original concentration of label. This corresponded to a 20-fold dilution of initially added ligand.

The efficiency of uptake after the gel filtration step was only about one-third of the normal uptake observed, reflecting the fragility of these vesicle preparations.

Gel Electrophoresis. Polyacrylamide gel electrophoresis was performed essentially following the procedure of Laemmli (1970) as detailed in Witzemann et al. (1983). Two-dimensional gel electrophoresis was performed according to O'Farrell (1975).

Labeling of the Vesicles with Azido-AMP or Azido-ATP. Either photolabeling of the vesicle proteins was carried out on fresh preparations (see Figure 5 and the two-dimensional analysis) as described above or the vesicles were pelleted and stored frozen at −20 °C. Although transport no longer occurred after thawing, the binding of the nucleotides did not change, according to the radiolabeling pattern seen upon polyacrylamide gel electrophoresis (see Figures 2–4 and 6). Labeling in this case was performed as follows:

Vesicles (~100 μg of protein) in 100 μL of buffer A were incubated with varying concentrations of azido-AMP or azido-ATP for 30 min at room temperature. In the case of azido-AMP, azido[^{3}H]AMP was dried down under nitrogen and resuspended in buffer A containing nonradioactive azido-AMP (molar ratio radioactive:nonradioactive 1:100). In the case of azido-ATP, azido[^{32}P]ATP was diluted with ATP in buffer A (molar ratio, azido[^{32}P]ATP:ATP 1:1000). These stock solutions were then added to the vesicles to give the final concentrations stated in the figure legends. After incubation, the vesicles were irradiated for 2 min at 254 nm on ice and then diluted 1:2 with sample buffer and electrophoresed. In some cases, the vesicles were first pelleted in a Beckman airfuge (100000*g* for 15 min) and resuspended in sample buffer before application to the gel.

Isolation of Mitochondrial ADP/ATP Carrier. *Torpedo* tissue, stored frozen in liquid nitrogen, was homogenized in buffer B [0.25 M sucrose, 0.5 mM ethylenediaminetetraacetic

acid (EDTA), 2.5 mM $MgCl_2$, and 20 mM Mops (4-morpholinepropanesulfonic acid), pH 6.8]. Mitochondria were pelleted 20 min at 10000 rpm in a Sorval SS34 rotor, followed by resuspension in buffer B. Mitochondrial ADP/ATP carrier was then isolated by using the procedure of Riccio et al. (1975a,b).

Analytical Methods. Protein was measured by the procedure of Bradford (1976) with bovine serum albumin as the standard. ATP was measured by the luciferin/luciferase method as modified by Dowdall et al. (1974). Enzymes were assayed by using the following procedures: lactate dehydrogenase, Johnson (1960); fumarase, Racker (1950); and cytochrome *c* oxidase, Smith (1955) as modified by Wharton & Tzagoloff (1967).

Results

Inhibition of Nucleotide Transport by Azido-AMP. Experiments were undertaken to determine whether the azido derivative of AMP inhibits the uptake of nucleotides into synaptic vesicles. Such inhibition would suggest that the analogue interacts directly with the nucleotide transport system. The uptake process in vitro has been characterized by Luqmani (1981) and shown to be fairly general, ATP, ADP, and AMP all being taken up by the same transport system. For this reason, we used both ATP and AMP azido derivatives in the course of our experiments as described below.

Samples of vesicles were incubated as described (Materials and Methods) in the presence of varying concentrations of azido-AMP. Vesicles were then incubated with radiolabeled ATP or AMP, and uptake was determined by gel filtration as described under Methods. Irradiation itself did not lyse the vesicles and had no effect on nucleotide transport.

The uptake of AMP was efficiently inhibited with increasing concentrations of azido-AMP as shown in Figure 1A. Each experimental point represents a single determination of the difference in AMP uptake between vesicles incubated and irradiated in the absence of azido-AMP (control = 100%) and vesicles which had been exposed to the azido analogue. The significance and reproducibiltiy of the inhibition studies are demonstrated by the fact that the various points (marked by different symbols in Figure 1A) were obtained from four different vesicle preparations assayed independently. Since the efficiency of uptake is related to the metabolic state of the vesicles after isolation, some variability is found between preparations [in 10 different experiments, the AMP transport in controls was 1.5 (±0.4 SD) pmol of AMP (nmol of ATP)$^{-1}$ min^{-1}]. The transport values at low azido-AMP concentrations were somewhat higher than the control values. This could be due to an irreversible inhibition of endogenous ATPases by the photolabel, or it may reflect the variation from sample to sample.

The uptake of the nucleotides can be described by classical Michaelis–Menten saturation kinetics, yielding apparent K_T (T symbolizes transport) ($=K_M$) values of about 3 mM for AMP. The apparent inhibition constant (K_i) for azido-AMP estimated from a Dixon-type plot for a competitive inhibitor was 2.9 mM (Figure 1B), suggesting that azido-AMP interacts with the same transport system as AMP or other nucleotides such as ATP, ADP, or GTP (Luqmani, 1981). This assumption was further supported by the finding that azido-AMP inhibited the uptake of ATP in experiments performed as described above. ATP was transported with a higher affinity (K_T = 1.12 mM) than AMP (or azido-AMP), and the inhibition was shifted toward higher concentrations of azido-AMP (not shown), supporting the conclusion that the adenine

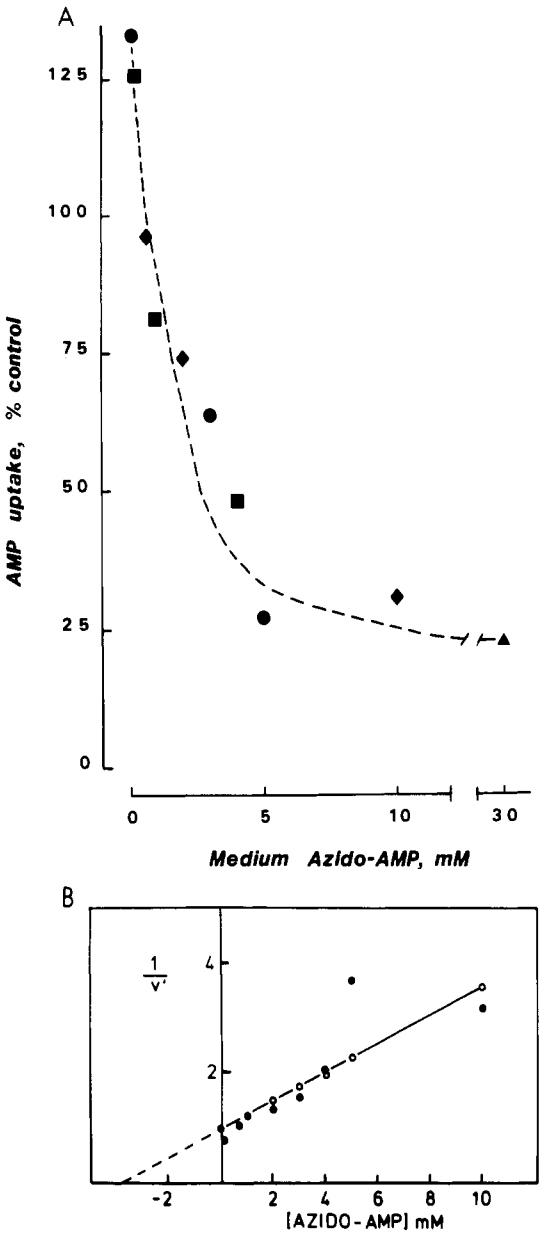

FIGURE 1: Inhibition of transport of [^{3}H]AMP and [^{3}H]ATP into vesicles by azido-AMP. Synaptic vesicle suspensions (1 mL, containing 100–150 μg of protein in 1 mM AMP, 0.85 M glycine, and 5 mM Hepes) were incubated for 30 min at 4 °C in the presence of varying concentrations of azido-AMP. The suspensions were then irradiated with UV light (254 nm) for 2 min on ice. [^{3}H]AMP (20 μM) was added and transport carried out as described under Materials and Methods. (A) Each symbol represents a single experiment. Uptake was calculated by first determining the nanomoles of AMP transported per milligram of protein and dividing by the total ATP concentration per milligram present in the vesicles to take into account vesicle integrity differing between samples. (B) The rate of uptake at 1 mM AMP and without inhibitor was v_0 = 1.5 (+0.4 SD) pmol of AMP (endogenous nmol of ATP)$^{-1}$ min^{-1}. The rates measured at increasing azido-AMP concentration were normalized with $v' = v$ (measured in the presence of azido-AMP)/v_0, and the reciprocal values were plotted vs. azido-AMP concentrations. The apparent inhibition constant K_i was estimated from a Dixon plot. Assuming K_T of AMP = 3 mM, we estimated a K_i of 2.9 mM for azido-AMP. The straight line fits to the experimental values (filled circles) and agrees very well with the theoretically predicted values (open circles).

nucleotides and their azido derivatives all share the same transport system.

The inhibition seen in Figure 1 may be due to a combination of covalent modification of the carrier from the UV irradiation and direct competition of the free azido-AMP with AMP for binding sites. It is therefore not possible to decide on the basis

Table I: Uptake of AMP Measured upon Photolysis of Vesicles in the Presence of N_3AMP[a]

expt	vesicles	UV irradiation, 2 min, 254 nm, 4 °C	Sephadex G-50 column	competitive inhibitor	rate of uptake (%)[b]
1	no label	+	→	no	100 (control)
2	+5 mM N_3AMP	no	→	no	100
3	+5 mM N_3AMP	+	→	+5 mM N_3AMP, 2-min irradiation, 254 nm	25
4	+5 mM N_3AMP	+	→	no	51
5	+2.5 mM N_3AMP	+	→	no	60
6	+10 mM N_3AMP	+	→	no	39

[a] Excess reagent and unreacted reagent were removed by filtration through a short Sephadex column. Experiments were performed by using two independent vesicle preparations. Uptake was done as described under Materials and Methods according to Luqmani (1981). [b] 100% uptake = 0.5 (±0.3 SD) pmol of AMP (nmol of ATP)$^{-1}$ min^{-1}.

of this result whether the transport system had been covalently modified. To investigate whether photolysis caused irreversible inhibition of AMP uptake, excess reagent had to be removed by a quick gel filtration step (see Materials and Methods). In these experiments, AMP uptake was not affected when the vesicles were photolyzed in the presence or absence of 5 mM AMP, excluding significant direct labeling due to photodecomposition of bound AMP. It was furthermore confirmed that the short column was sufficient to reduce excess label to a level where competitive inhibition of AMP uptake would be negligible. Incubation of vesicles with 5 mM photolabel in the dark had no effect on AMP uptake. Upon photolysis, however, this uptake was significantly reduced due to irreversible inhibition by the covalently bound photolabel (Table I).

To get some estimate of the efficiency of the photolabeling process, 5 mM N_3AMP (irradiated for 2 min at 254 nm) was added back to photolabeled vesicles prior to AMP uptake (Table I, experiment 3). In this case, uptake had been reduced to about 25% of the control, which is in agreement with the results of the above-described studies (Figure 1). Comparison with the uptake of photolabeled vesicles (51% in the absence of additional free ligand) indicated that the labeling efficiency was ≥50%.

Labeling of Vesicle Polypeptides with Azido-AMP and Azido-ATP. Vesicles were isolated on a linear sucrose gradient as in the previous experiments and employed in the labeling experiments described below. The more highly purified vesicles obtained by zonal centrifugation were not used because of low yield and because these vesicles do not transport nucleotides, a fact which suggests damage to the vesicles during isolation (P. Giompres, personal communication). Isolated vesicles were incubated with azido[^{3}H]AMP at concentrations of 1 and 10 mM, washed, and analyzed by sodium dodecyl sulfate (NaDodSO$_4$)–polyacrylamide gel electrophoresis. Figure 2 shows the labeling pattern seen with the azido label at the two concentrations. In both cases, substantial labeling was found on a large number of polypeptides, the pattern of which was very similar at both azido[^{3}H]concentrations. The only striking difference between the two conditions was the labeling of a polypeptide of M_r 34 000. If one assumes the K_i of transport (2.9 mM) reflects the affinity of the ligand for its binding site, one would expect a large difference in the labeling of the translocase between 1 and 10 mM azido-AMP. Indeed, the fluorogram showed a large increase of radioactivity bound to the M_r 34 000 band. This is the only protein to show such stimulation. The radiolabeled M_r 34 000 polypeptide corresponded to the prominent polypeptide seen upon Coomassie brilliant blue staining (Figure 2c) which is thought to be a vesicle-specific component (Stadler & Tashiro, 1979). The

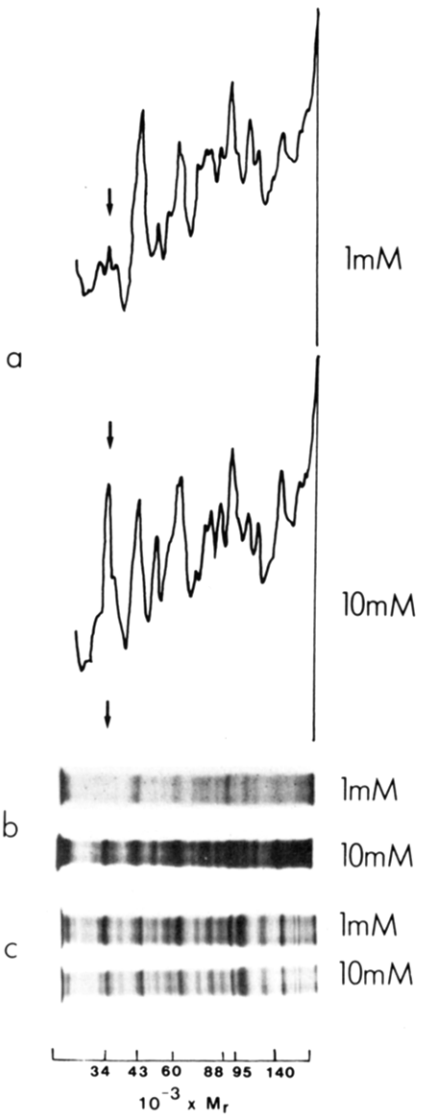

FIGURE 2: Labeling of vesicular proteins with azido[^{3}H]AMP. Vesicles (100 μg of protein) isolated on a linear sucrose gradient were incubated with azido[^{3}H]AMP (1 and 10 mM) for 30 min at room temperature and then irradiated for 2 min on ice. The vesicles were pelleted for 15 min at 100000*g* in a Beckman airfuge, resuspended in electrophoresis sample buffer, and examined by using NaDodSO$_4$–polyacrylamide gel electrophoresis. (a) Scan of the fluorogram; (b) fluorogram; (c) Coomassie brilliant blue stained polypeptides. The arrow indicates the position of the M_r 34 000 polypeptide.

labeling of another protein of M_r 43 000 (thought to be actin due to its migration behavior and isoelectric properties on one- and two-dimensional gel electrophoresis) showed a much

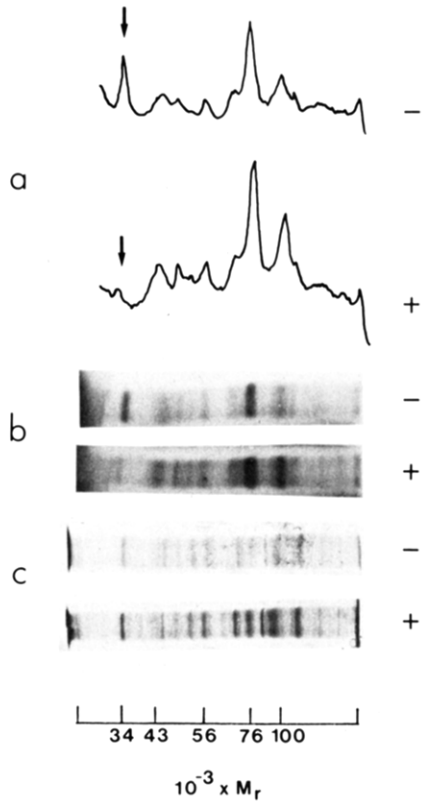

FIGURE 3: Labeling of vesicles with 1 mM azido[^{32}P]ATP after a preincubation with 2 mM azido-AMP. Vesicles (100 μg of protein), after preincubation and irradiation with (+) and without (–) unlabeled azido-AMP at 2 mM, were incubated with azido[^{32}P]ATP at 1 mM. The samples were irradiated and electrophoresed (8.5% acrylamide) as described under Materials and Methods. Molecular weight standards consisted of the following: myosin (M_r 200 000), β-galactosidase (M_r 116 250), phosphorylase *b* (M_r 92 500), BSA (M_r 66 200), ovalbumin (M_r 45 000), and carbonic anhydrase (M_r 31 000). (a) Density scan of the autoradiogram; the molecular weights of the major radiolabeled polypeptides are indicated; (b) autoradiogram; (c) Coomassie brilliant blue stained polypeptides. Arrows indicate the position of the M_r 34 000 polypeptide.

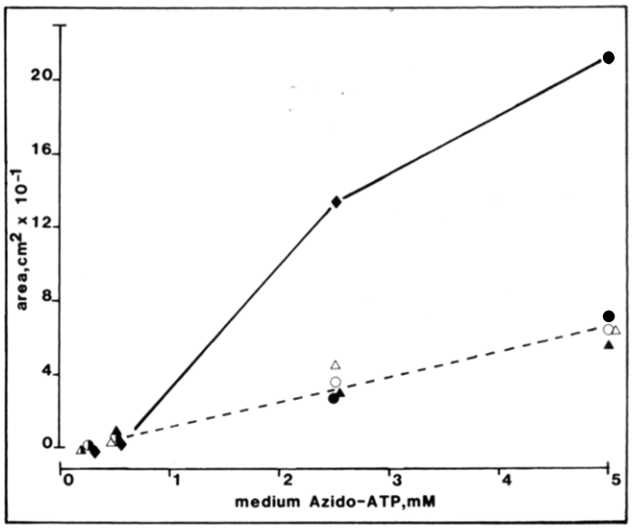

FIGURE 4: Labeling of vesicles with increasing concentrations of azido[^{32}P]ATP. Vesicles (100 μg of protein) were incubated with increasing concentrations of azido[^{32}P]ATP as described. Gels were exposed to Kodak Safety Film ARO. Autoradiograms were scanned, and the area under the peaks was calculated by using the formula $A = {}^1/_2bh$. The five most prominent bands had the following molecular weights: (○) 100 000; (●) 76 000; (△) 64 000; (▲) 43 000; (♦) 34 000. Molecular weight standards were as in Figure 3.

smaller increase in bound radioactivity, probably reflecting nonspecific labeling. Experiments were carried out with azido[^{3}H]AMP so that the binding results could be compared directly to the transport data in Figure 1.

In the following experiments, we tested whether photolabeling using azido[^{32}P]ATP would also result in a selective modification of vesicular proteins. Figure 3 shows the vesicle polypeptides after labeling. When vesicles were incubated and photolyzed with 1 mM azido[^{32}P]ATP, the same M_r 34 000 polypeptide seen before upon azido[^{3}H]AMP labeling was strongly labeled. ATP and, therefore, probably azido-ATP display a higher affinity ($K_T \sim 1$ mM) for the transport system than AMP ($K_T \sim 3$ mM), which explains the efficient labeling of the M_r 34 000 polypeptide at concentrations as low as 1 mM. The labeling of the M_r 34 000 band could be specifically eliminated by pretreating the vesicles with nonradiolabeled azido-AMP (Figure 3b). The radioactivity bound to the M_r 34 000 polypeptide was substantially reduced while all other polypeptides showed increased radiolabeling, probably due to the slightly higher amounts of protein present (Figure 3c).

Direct labeling of the nucleotide binding proteins was attempted by UV irradiation of vesicles which had been incubated in millimolar [^{3}H]ATP concentrations. Irradiation times were 10 min at 254 nm. Under these conditions, little or no radiolabel was bound, indicating that the incorporation of radioactivity in our experiments which used only 2-min irradiation time was solely due to the reaction of the photoactivated nitrene residues attached to the radiolabeled nucleotides. This is in agreement with the transport results. Irreversible inhibition could be observed only upon photolysis in the presence of azido derivatives but not in the presence of AMP or ATP.

Further evidence for the selective radiolabeling of a single polypeptide is shown in Figure 4. Vesicles were photolyzed in the presence of increasing concentrations of azido[^{32}P]ATP. The autoradiogram was quantitiated by densitometry, and the area under various peaks was plotted against azido[^{32}P]ATP concentrations for the five most prominent bands. As can be seen, the increase in labeling for four of the proteins is linear and therefore reflects nonspecific labeling. The linear increase in bound radioactivity indicated in addition that the efficiency of the photolabeling process was not significantly reduced due to limiting absorption of incident light by nucleotide levels up to 5 mM. However, the increase in radioactivity of the M_r 34 000 protein was much higher and, correcting for nonspecific labeling, appeared to be saturable at azido[^{32}P]ATP concentrations above 5 mM.

Labeling of Polypeptides across a Linear Gradient. To demonstrate the association of the M_r 34 000 polypeptide identified above with vesicles, proteins separated on a linear sucrose gradient were labeled with azido[^{32}P]ATP at concentrations of 0.5 and 2 mM. The vesicle translocase should fulfill the following criteria in such an experiment: it should be enriched in the vesicle-containing fractions, and it should label strongly at 2 mM and to a much lower extent at 0.5 mM azido[^{32}PATP. The ATP content was used to locate the vesicle-containing fractions obtained after centrifugation on a linear sucrose gradient. As shown in Figure 5A, soluble ATP was found in the top fractions, 15 and 16, and vesicular ATP in fractions 6–12. As demonstrated in Figure 5C, the M_r 34 000 protein was efficiently labeled at 2 mM azido[^{32}P]ATP concentrations and highly enriched in the vesicle-containing fractions. Labeling was seen in addition in fractions 3–5. It should be mentioned that the vesicle fractions obtained from sucrose zonal centrifugations display some heterogeneity.

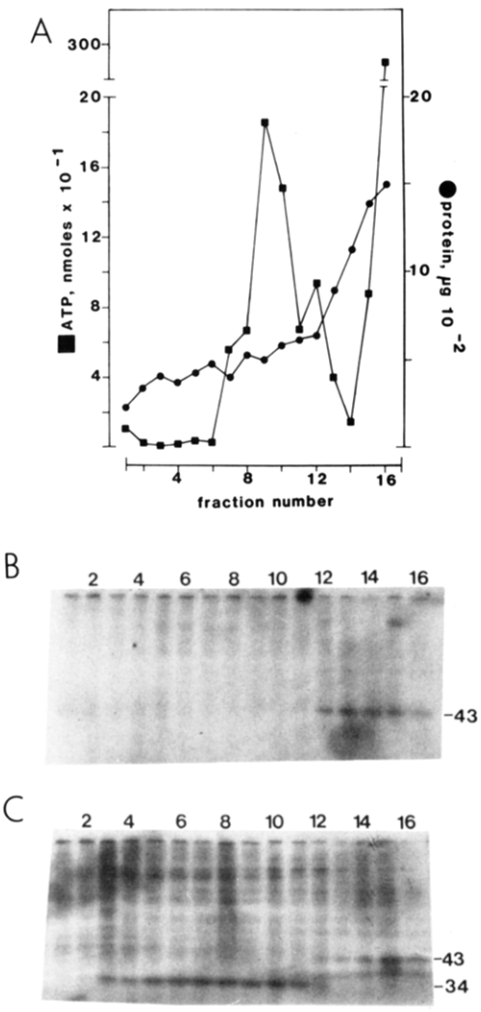

FIGURE 5: Labeling of vesicular proteins separated on a linear sucrose gradient. (A) Vesicles were isolated on a linear sucrose density gradient as described. ATP served as a marker of the vesicle-containing fractions. 100 µg of protein from each fraction was incubated with 0.5 (B) or 2 mM (C) azido[^{32}P]ATP. After irradiation, protein was electrophoresed on a 12.5% NaDodSO$_4$–polyacrylamide gel. Numbers given in (B) and (C) are $M_r \times 10^{-3}$ and indicate the position of the M_r 34 000 polypeptide and of actin (apparent M_r in this gel system 43 000). The density of the gradient decreases from fraction 1 to 16.

While the majority of the vesicles coincided with the ATP distribution, it was found that a second population of denser vesicles existed containing much less ATP. On electrical stimulation, which leads to the release of neurotransmitter and ATP but also to the reuptake of newly synthesized extracellular material, it was demonstrated that it is this denser vesicle population which contains most of the newly synthesized ATP and acetylcholine (Zimmerman & Denston, 1977). In our gradients, the vesicles would not be separated as two distinct populations, but the denser vesicles would trail off from the major ATP-containing fractions. The labeling seen in fractions 3–5 is therefore most likely representative of this smaller dense vesicle fraction, lending additional support for the specificity of the photolabeling. No other radiolabeled polypeptide showed such a characteristic distribution. Actin (M_r 43 000 in this gel system) was also labeled, but the majority did not comigrate with the vesicle fractions and was found in the top fractions of the gradient. Higher molecular weight proteins ($M_r \geq 100000$), on the other hand, were associated with membranes of higher density than the vesicles and could originate from mitochondrial contaminations (see below). When the gradient fractions were subjected to azido[^{32}P]ATP labeling at 0.5 mM (Figure 5B), most of the polypeptides which had been labeled at 2 mM had again incorporated radioactivity, but no radioactivity bound to the M_r 34 000 polypeptide was detectable.

M_r 34 000 Polypeptide Is Not a Mitochondrial Contaminant. The labeling pattern seen with vesicles from the linear sucrose gradients might also arise partly from the contamination of the vesicles with mitochondrial proteins. The mitochondrial carrier has a high affinity for azido-ATP (Schäfer et al., 1976) and is of similar size (M_r 30 000). Therefore, it was important to test whether our vesicle preparation was contaminated with mitochondrial membranes. First, assaying for cytochrome *c* oxidase (a marker for inner mitochondrial membranes), we estimated the amount of contamination from mitochondrial-derived membrane fragments across the linear sucrose gradient (Figure 6A). The enzyme activity was normalized with respect to the activity (being set equal to 1.0) found in the crude mitochondrial pellet obtained in the course of the vesicle preparation (see Materials and Methods). As can be seen, there is virtually no contamination of the vesicles with cytochrome *c* oxidase (less than 0.028; in other gradients examined, none showed an activity higher than 0.04). Therefore, a significant contamination of the vesicles with mitochondrial proteins is unlikely, certainly not enough to account for the large amount of the M_r 34 000 protein seen with the Coomassie brilliant blue stain (Figures 2 and 3). As a further control, the mitochondrial ADP/ATP carrier was isolated from *Torpedo* mitochondria by using the procedure of Riccio et al. (1975a,b) and incubated with azido-ATP (10 mM) (Figure 6B). This protein (lane b) can be clearly separated on a one-dimensional 12.5% NaDodSO$_4$–polyacrylamide gel from the larger protein labeled in vesicles (lane a). Therefore, we can conclude that the M_r 34 000 protein that is labeled with the azido nucleotides is a vesicle-specific component and not contaminating ADP/ATP carrier from the mitochondria.

Analysis of the M_r 34 000 Protein on Two-Dimensional Gel Electrophoresis. Vesicles were photolabeled and subjected to two-dimensional gel electrophoresis as described by O'Farrell (1975). This showed that all of the radioactivity associated with the M_r 34 000 polypeptide focused as a single component at about pH 7.7. A similar migration behavior has been described for the V11 component (Tashiro & Stadler, 1978), which may indicate that the ATP translocase identified here is identical with a major protein of the vesicles (V11). As already demonstrated by one-dimensional gel electrophoresis, the labeling of this polypeptide was significantly reduced at 0.5 mM azido[^{32}P]ATP, while the other radiolabeled polypeptides did not show this strong concentration-dependent decrease in labeling efficiency.

Discussion

Nitrenes generated from azides upon photolysis are short-lived and can react with a variety of chemical functions in a relatively indiscriminate manner (Bayley & Knowles, 1977). When bound to a specific site of a protein, photolysis leads to covalent incorporaiton of the nitrene. Vesicle preparations analyzed upon NaDodSO$_4$–polyacrylamide gel electrophoresis after they had been subjected to UV irradiation in the presence of radiolabeled azido-AMP or -ATP revealed that several proteins bound the photolabile nucleotide derivatives. This was, however, expected since these labels not only could interact at specific binding sites, such as the ATP sites of actin, but also react less specifically due to ionic or hydrophobic associations with proteins. Unspecific labeling seemed to be furthermore inevitable at the increased concentrations of photolabel used in these experiments.

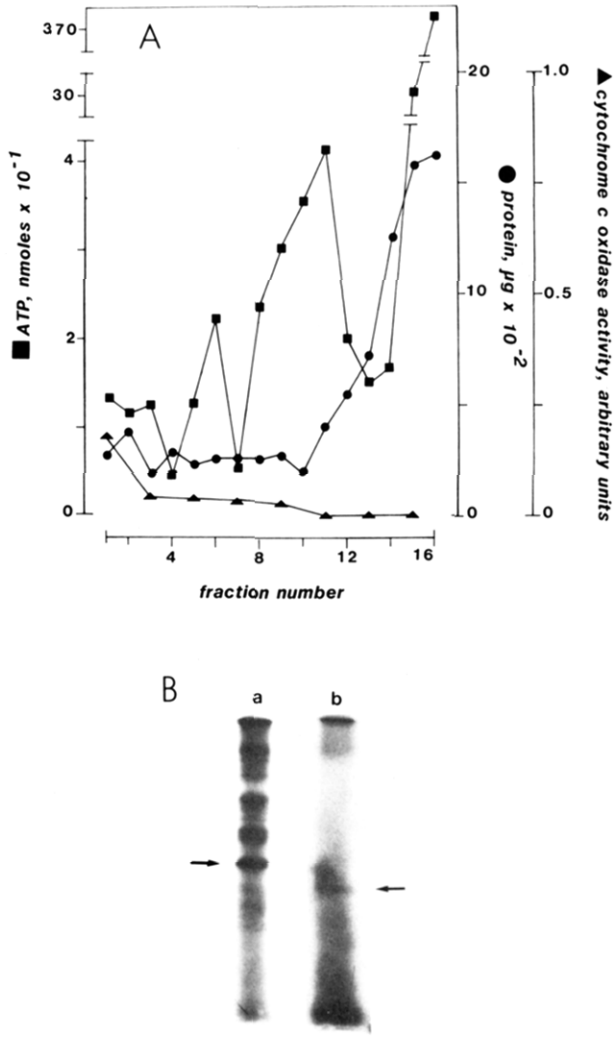

FIGURE 6: Comparison of labeling by vesicles and the isolated ADP/ATP carrier from *Torpedo* mitochondria. (A) Vesicles were isolated on a linear sucrose gradient and located by assay of ATP. 50 μL of each fraction, as well as the mitochondria-enriched pellet generated in the initial vesicle isolation procedure and resuspended in buffer A, was assayed for cytochrome *c* oxidase activity. Activity in each gradient fraction was normalized with respect to the activity found in the crude mitochondria-enriched pellet (=1). (B) The ADP/ATP carrier was isolated from *Torpedo* mitochondria by using the procedure of Riccio et al. (1975a,b). Vesicles (50 μg) and ADP/ATP carrier (5 μg) were incubated with 2.5 mM azido[^{32}P]ATP as before. Because the ADP/ATP carrier had been isolated in 4% Triton, Triton was added to the vesicles prior to labeling to a final concentration of 4%. (a) Vesicles; the arrow indicates the position of the M_r 34 000 polypeptide; (b) mitochondrial ADP/ATP carrier; the arrow indicates the position of the mitochondrial carrier of M_r 30 000.

A selective concentration-dependent photolabeling occurred, however, on a polypeptide of M_r 34 000. The incorporation of radioactivity into the M_r 34 000 polypeptide increased dramatically between 0.5 and 5 mM azido-ATP, and the M_r 34 000 polypeptide became the most extensively labeled component. The dependence on the concentration of photolabel present corresponded closely to the concentration range found to be optimal for ATP transport. There is good evidence supporting the notion that the translocase not only binds azido-ATP (-AMP), as indicated by the uptake inhibition studies, but also can be covalently modified by these photoaffinity reagents: AMP uptake appeared to be irreversibly and efficiently inhibited after the vesicles had been photolyzed in the presence of azido-AMP.

Additional evidence of a specific labeling process is that no irreversible inhibition of uptake or incorporation of radioactivity occurred without UV irradiation. The irradiation itself had no effect on transport and therefore on the binding properties of the proteins involved in transport, and it did not affect the polypeptide pattern observed upon NaDodSO$_4$–polyacrylamide gel electrophoresis. Photoaffinity labeling of vesicles using the nonradiolabeled azido-AMP prevented subsequent incorporation of radioactivity only into the M_r 34 000 polypeptide, while other polypeptides showed no significant differences. Direct labeling due to UV-induced photodecomposition of bound nucleotides was not detected.

The radiolabeled M_r 34 000 polypeptide had the same molecular size as the major Coomassie brilliant blue stained component of our vesicle preparations. When the labeling pattern of proteins separated on sucrose density gradients was examined, this protein was labeled strongly at 2 mM and only weakly at 0.5 mM azido-ATP concentrations and was highly enriched in the vesicle fractions. The direct comparison with the labeling efficiency and distribution of nonexclusively vesicle-associated proteins such as actin gives further support for the selective photolabeling of the M_r 34 000 polypeptide and its specific enrichment in vesicular fractions. First, actin was strongly labeled by azido-ATP but did not display a significant concentration-dependent effect. Second, while the bulk of the actin was not associated with vesicles and was found together with the soluble protein fraction, a small amount of actin remained bound to the vesicle fraction, possibly representing the vesicle-specific form of actin described by Zechel & Stadler (1982).

The results of two-dimensional gel electrophoresis revealed that a single polypeptide (pI ~ 7.7) had been labeled by azido-ATP. Similar electrophoretic migration behavior has been described for the vesicle component V11 (Tashiro & Stadler, 1978), suggesting that the two proteins may be identical. On the basis of our uptake inhibition studies and the selective photolabeling results, one can conclude that the M_r 34 000 protein is directly involved in ATP transport into synaptic vesicles. A protein of similar molecular weight has been shown to exist in *Torpedo californica* and *Narcine brasiliensis* vesicles (Wagner et al., 1978).

It is interesting to note that in the ATP transport mechanisms so far examined all have been characterized by high K_T values (except for the mitochondrial carrier). Kostron et al. (1977) have identified a carrier-mediated transport activity in isolated bovine chromaffin granules which shows saturation at concentrations over 2 mM ATP and has a K_T of 1.4 mM. Later work by Carmichael et al. (1980) demonstrated similar K_T values for chromaffin granules isolated from pig (1.37 mM), horse (0.89 mM), and ox (1.2 mM). In an entirely different system, Chaudry & Baue (1980) have demonstrated an ATP uptake system in intact rat soleus muscle and hemidiaphragms (saturation at around 2 mM). The relatively low affinities of these systems suggest that they might serve as control points in the process of vesicle recycling.

To the best of our knowledge, the synaptic vesicle translocase is the first ATP transport protein to have been identified other than the mitochondrial ADP/ATP carrier. While there seem to be some similarities with the mitochondrial carrier (size, isoelectric point, transport of nucleotides), it should be pointed out that the functional properties are quite different (i.e., high K_T values, low V_{max}, low substrate specificity, and its function as an accumulator rather than as an exchanger). Further identification of other nucleotide carriers such as the chromaffin granule and the rat soleus muscle translocases will be needed to understand the structural and functional relationship

between nucleotide transport systems.

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Registry No. ATP translocase, 9068-80-8; azido-AMP, 60731-47-7; azido-ATP, 53696-59-6; ATP, 56-65-5; AMP, 61-19-8.

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Phosphorus-31 Nuclear Magnetic Resonance Investigation of Membrane Vesicles from *Escherichia coli*†

Arthur G. Hunt,‡ Virgil Simplaceanu, Jen-Shiang Hong,* and Chien Ho*

ABSTRACT: Phosphorus-31 nuclear magnetic resonance studies of isolated membrane vesicles prepared from *Escherichia coli* PSM116 as described by Hunt and Hong [Hunt, A. G., & Hong, J.-S. (1981) *J. Biol. Chem. 256*, 11988–11991; Hunt, A. G., & Hong, J.-S. (1983) *Biochemistry 22*, 844–850] are detailed here. This strain harbored a recombinant plasmid containing the phosphoglycerate transport system from *Salmonella typhimurium* (pJH7). Evidence indicating a surprising metabolic diversity, such as the presence of the enzymes enolase and phosphoglycerate mutase, is presented. The nature of the energization of these membrane vesicles for transport as described by Hugenholtz et al. [Hugenholtz, J., Hong, J.-S., & Kaback, H. R. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3446–3449] is also discussed. Membrane vesicles prepared from the PSM116 strain do not form a transmembrane pH gradient when phosphoenolpyruvate is added. The present results show that phosphorus-31 nuclear magnetic resonance spectroscopy is an excellent tool to investigate the metabolism of membrane vesicles.

In recent years, nuclear magnetic resonance (NMR)[1] spectroscopy has proven to be a useful technique for studying bioenergetics and metabolism in intact cells and tissues [for example, see Lam et al. (1979), Burt et al. (1979), Shulman

† From the Department of Cell Physiology, Boston Biomedical Research Institute, Boston, Massachusetts 02114 (A.G.H. and J.-S.H.), and the Department of Biological Sciences, Carnegie-Mellon University, Pittsburgh, Pennsylvania 15213 (V.S. and C.H.). *Received June 22, 1983.* Supported by research grants from the National Institutes of Health (GM-26874 and GM-29843) and the National Science Foundation (PCM 82-08829).

‡ Present address: Laboratory of Plant Molecular Biology, Rockefeller University, New York, NY 10021.

[1] Abbreviations: NMR, nuclear magnetic resonance; PEP, phosphoenolpyruvate; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; ATP, adenosine 5′-triphosphate; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; 2-PGA, 2-phosphoglyceric acid; 3-PGA, 3-phosphoglyceric acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; pgt, phosphoglycerate transport; EDTA, ethylenediaminetetraacetic acid; MDPA, methylenediphosphonic acid; DCCD, *N,N′*-dicyclohexylcarbodiimide; PMS, phenazine methosulfate.

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